

Mechanisms by Which Psychologic Stress Alters Cutaneous Permeability Barrier Homeostasis and Stratum Corneum Integrity

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Although many skin disorders, including psoriasis and atopic dermatitis, are adversely affected by psychologic stress (PS), the pathophysiologic link between PS and disease expression remains unclear. Recent studies demonstrated PS-induced alterations in permeability barrier homeostasis, mediated by increased endogenous glucocorticoids. Here, we assessed the mechanisms by which PS alters stratum corneum (SC) function. Insomniac psychologic stress (IPS) altered both barrier homeostasis and SC integrity. IPS decreased epidermal cell proliferation, impaired epidermal differentiation, and decreased the density and size of corneodesmosomes (CD), which was linked to degradation of CD proteins (e.g., desmoglein1). Barrier compromise was linked to decreased production and secretion of lamellar bodies (LB), which in turn could be attributed to a decrease in *de novo* synthesis of epidermal lipids. Topical physiologic lipids (equimolar cholesterol, ceramides, and free fatty acids) normalized both barrier homeostasis and SC integrity in IPS mice, further evidence that lipid deficiency accounted for these functional abnormalities. Thus, PS inhibition of epidermal lipid synthesis results in decreased LB formation and secretion, as well as decreased CD, compromising both permeability barrier homeostasis and SC integrity. These studies suggest that topical treatment with epidermal physiologic lipids could be beneficial in stress-induced, barrier-associated dermatoses, such as psoriasis and atopic dermatitis.

Key words: corneodesmosome/epidermal lipid synthesis/lamellar body/stratum corneum integrity/transepidermal water loss

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Psychologic stress (PS) is well recognized to provoke, exacerbate, and propagate many cutaneous dermatoses associated with abnormal epidermal barrier function, such as psoriasis and atopic dermatitis (Rostenberg, 1960; Ghadially *et al*, 1996; Gupta and Gupta, 1996; Tausk and Nousari, 2001; Proksch *et al*, 2003; Sugarman *et al*, 2003). It is also well recognized that optimal management of these disorders mandates consideration, and where possible mitigation of co-existent emotional stressors. For example, deployment of stress-reduction techniques (e.g., meditation, biofeedback, and hypnosis) clearly benefits some patients with stress-associated dermatosis (Gaston *et al*, 1991; Farber and Nall, 1993; Kabat-Zinn *et al*, 1998; Shenefelt, 2000). Based upon extensive studies on the immune and neuroendocrine systems, the prevailing view holds that immune and neuroendocrine mechanisms account for the negative effects of PS on skin (O'Sullivan *et al*, 1998). Recent studies have shown that various types of PS compromise permeability barrier function in humans (Altemus *et al*,

2001; Garg *et al*, 2001). Furthermore, in the case of examination-induced PS, the alterations in barrier homeostasis were proportional to the extent of PS (Garg *et al*, 2001). Thus, PS-induced alterations in barrier function could represent a clinically relevant mechanism that contributes to disease expression.

Parallel studies in rodent models have provided insights about the mechanisms leading to PS-induced alterations in barrier homeostasis. First, the PS-induced barrier abnormalities could be reversed by co-administered sedatives, such as chlorpromazine or diazepam (Denda *et al*, 1998, 2000). Moreover, PS adversely affects barrier homeostasis by stimulating increased endogenous production of glucocorticoids (GC) (Denda *et al*, 2000). Co-administration of the GC receptor antagonist, RU 486, blocked emergence of the PS-induced abnormalities in barrier homeostasis. Subsequent studies demonstrated directly the negative consequences of GC on both barrier function and stratum corneum (SC) integrity (Kao *et al*, 2003).

Recent studies have begun to elucidate the mechanisms by which increased GC perturbs these epidermal functions. Prior work has shown that long-term GC treatment decreases epidermal proliferation and differentiation (Laurence and Christophers, 1976; du Vivier *et al*, 1982; Sheu *et al*, 1991; Sheu *et al*, 1997). But even short-term GC treatment inhibits epidermal lipid synthesis, resulting in de-

Abbreviations: CD, corneodesmosome; DSG1, desmoglein1; EM, electron-microscope; GC, glucocorticoid; IPS, insomniac psychologic stress; LB, lamellar body; PCNA, proliferation cell nuclear antigen; PPM, parts per million; PS, psychologic stress; SC, stratum corneum; SG, stratum granulosum; TEWL, transepidermal water loss; TUNEL, TdT-mediated dUTP nick end-labeling

creased production and secretion of lamellar bodies (LB), and impaired production of lamellar membranes in the SC (Kao *et al*, 2003). Epidermal *de novo* synthesis of cholesterol, fatty acids, and ceramides is required for the formation of LB (Grubauer *et al*, 1987; Feingold *et al*, 1990; Feingold, 1991; Holleran *et al*, 1991; Mao-Qiang *et al*, 1993). These lipids are then packaged into LB, whose secretion restores the extracellular lamellar membranes that mediate SC barrier function (Menon *et al*, 1992). Finally, topical treatment with a mixture of physiologic lipids, which mimic SC lipid composition, normalizes permeability barrier homeostasis in GC-treated mice, linking the GC-induced reduction in epidermal lipid synthesis to the delay in barrier recovery (Kao *et al*, 2003).

Although the mechanistic links between increased GC and barrier homeostasis are becoming clearer, how PS itself alters epidermal function remains unclear. Based on our observations in GC-treated animals, we hypothesized and then showed here that PS, similar to GC, inhibit epidermal lipid synthesis, resulting in a decline in the production and secretion of LB, coupled with a reduction in the amounts of lamellar membranes in the SC interstices. Additionally, we showed that PS, like GC, also decreases SC integrity. Finally, we found that provision of exogenous physiologic lipids overrides the negative effects of PS on epidermal function, restoring both permeability barrier homeostasis and SC integrity to normal, even in the face of ongoing PS.

Results

Insomniac psychological stress (IPS) compromises epidermal barrier homeostasis and SC integrity Prior studies have shown that neither immobilization nor crowding alter basal transepidermal water loss (TEWL) (Denda *et al*, 1998, 2000). Therefore, we first assessed this functional parameter in IPS and control animals, and, as expected basal barrier function did not differ (8.83 ± 0.67 parts per million (PPM) per cm^2 per h in IPS animals vs 8.89 ± 0.75 PPM per cm^2 per h in control). Moreover, there were no alterations in surface pH and hydration (data not shown). Yet, as with other forms of PS (Denda *et al*, 2000), IPS also delayed barrier recovery significantly following acute barrier disruption (Fig 1). Thus, IPS, like other forms of PS, significantly alters the kinetics of barrier recovery after acute insults.

Prior studies of PS in mice have not, however, assessed potential alterations in SC integrity, i.e., SC resistance to mechanical insults. As shown in Fig 2, SC integrity was significantly compromised in IPS animals. Although TEWL levels remained comparable in both PS and control groups over the first three tape strippings, significant differences began to emerge with subsequent strippings (#4–5). Thus, short-term IPS appears to compromise SC integrity of deeper layers with the SC.

Structural bases for IPS-induced functional deficits To determine whether the barrier and integrity abnormalities induced by IPS are because of alterations in epidermal structure, we next assessed epidermal proliferation by proliferation cell nuclear antigen (PCNA) staining and epidermal

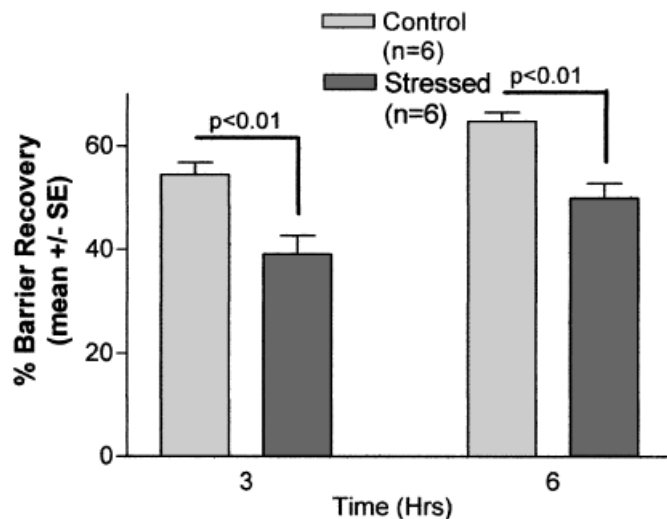


Figure 1
Effect of psychological stress on barrier recovery after acute disruption. Insomniac psychological stress delayed barrier recovery that was measured at 3 and 6 h after tape stripping. Statistical analysis was performed using repeated ANOVA. Results are shown as mean \pm SE (n = 6 animals in each group).

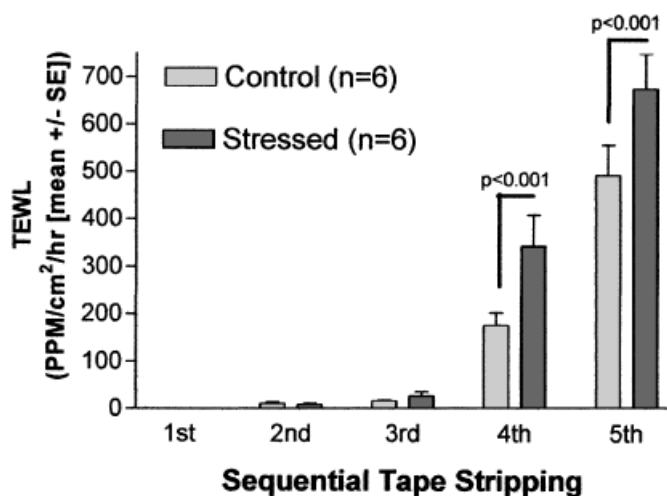


Figure 2
Effect of psychological stress on stratum corneum (SC) integrity. SC integrity was reduced in insomniac psychological stress animals after the third tape stripping. Statistical analysis was performed using repeated ANOVA.

thickness. Whereas short-term IPS did not significantly affect epidermal thickness in comparison with controls (63 ± 5.7 vs 60 ± 2.5 μm), PCNA-positive cells declined by 25% in IPS animals in comparison with controls (Fig S1). In contrast, IPS did not alter TdT-mediated dUTP nick end-labeling (TUNEL) staining in IPS mice (not shown). These results indicate that short-term IPS inhibits keratinocyte proliferation, but under such short-term conditions, PS was not yet sufficiently sustained to lead to epidermal thinning.

We next examined the effects of IPS on the expression of the epidermal differentiation-related proteins, involucrin, loricrin, and filaggrin. Immunostaining for each of these three proteins declined in the epidermis of IPS mice despite the long half-lives of these proteins (Fig S2). Thus, short-

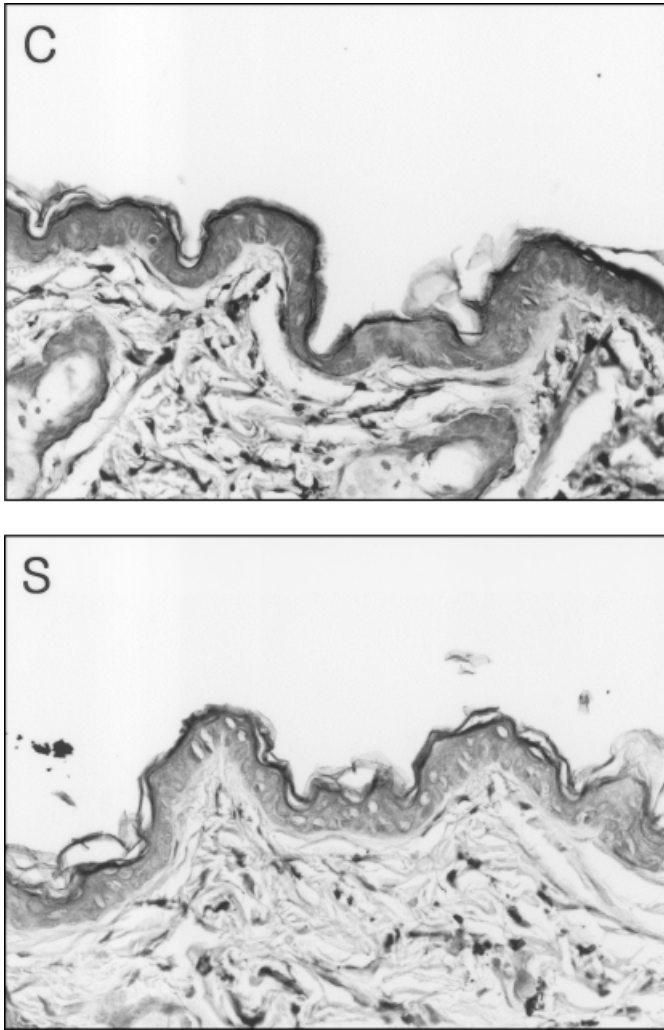


Figure 3
Effect of psychologic stress on desmoglein1 (DSG1) expression. DSG1 immunohistochemical staining was decreased in insomniac psychologic stress animals (S) compared with controls (C).

term IPS decreased not only epidermal proliferation but also the expression of epidermal differentiation-related proteins.

To begin to assess the basis for the SC integrity abnormality, we first assessed desmoglein1 (DSG1) immunohistochemical staining, and second the ultrastructural appearance of individual corneodesmosomes (CD) in the lower SC. DSG1 staining was decreased in IPS group compared with controls (Fig 3). Many fragmented and shortened CD were found in the lower SC of IPS mice in comparison with control. Quantitative electron microscopic (EM) analysis confirmed that CD density was significantly decreased in IPS mice (Fig 4). These results show that the emergence of an abnormality in SC integrity correlates with a diminution in both the size and number of CD, further linked to degradation of CD proteins (e.g., DSG1) in the lower SC.

We next examined the mechanisms by which IPS delays barrier recovery, initially assessing the effects on SC intercellular lamellae. The amount of lamellar membranes was decreased in IPS group, which appeared as thinner membranes compared with normal control (Fig 5). Next, we assessed the effects of IPS on the LB secretory system. In IPS animals there was an apparent reduction in the number

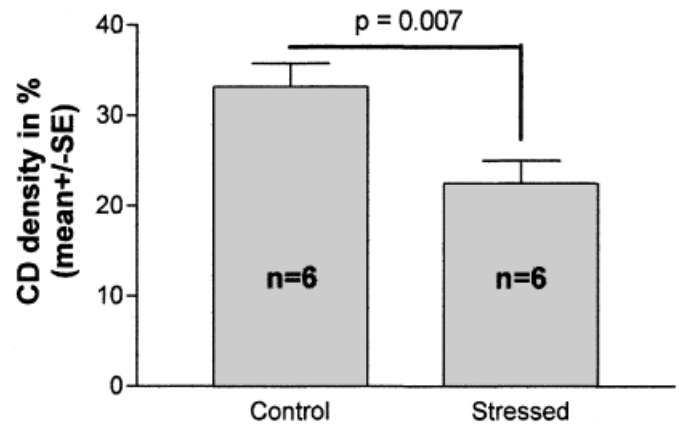


Figure 4
Effect of psychologic stress on corneodesmosome density. Quantitative electron microscopic (EM) analysis for corneodesmosomes (CD) density showed a significant decrease in CD density in insomniac psychologic stress mice. Statistical analysis was performed using unpaired Student's *t* test. Results are shown as mean \pm SE ($n=5$).

(density) of LB in the cytosol of stratum granulosum (SG) cells in IPS animals (Figs 6A and 7A). Moreover, there was also both an apparent as well as quantitative decrease in the amount of secreted lamellar contents at the SG–SC interface in IPS animals (Figs 6A and 7A). Following acute barrier disruption, a process that normally stimulates LB production and secretion (Menon *et al*, 1992), the number (density) of LB remained reduced in the cytosol of SG cells of IPS animals (Figs 6B and 7B). Furthermore, the amount of secreted lamellar material at the SG–SC interface in IPS animals remained significantly lower than in controls (Figs 6B and 7B). Thus, the production of LB, not only in the basal state but also following acute barrier disruption, is decreased in IPS, and as a result the amount of secreted material delivered to the SC interstices is likewise reduced.

IPS inhibits epidermal lipid synthesis Since the formation of nascent LB requires *de novo* epidermal lipid synthesis (Feingold *et al*, 1990; Holleran *et al*, 1991; Mao-Qiang *et al*, 1993), we next assessed whether the IPS-induced decrease in LB production was because of suppression of epidermal lipid synthesis. As seen in Fig 8, epidermal cholesterol and fatty acid synthesis are reduced by about 50% in comparison with control under basal conditions, and epidermal ceramides by about 35%. These results show that IPS inhibits the synthesis of the key constituent lipids of LB.

Topical lipids override IPS-induced abnormalities in barrier function and SC integrity To further assess whether the reduction in epidermal lipid synthesis in IPS animals accounts for the IPS-induced functional abnormalities, we next determined whether topical provision of the inhibited lipids would override the abnormalities in barrier recovery and SC integrity. As shown in Fig 9, a single topical treatment with an equimolar lipid mixture of cholesterol, free fatty acids, and ceramides, a lipid mixture that has no effect on barrier recovery rate in normal mice (Mao-Qiang *et al*, 1995; Man *et al*, 1996), markedly accelerated barrier recovery in IPS animals. Additionally, topical treatment with these lipids during IPS also reversed the IPS-induced

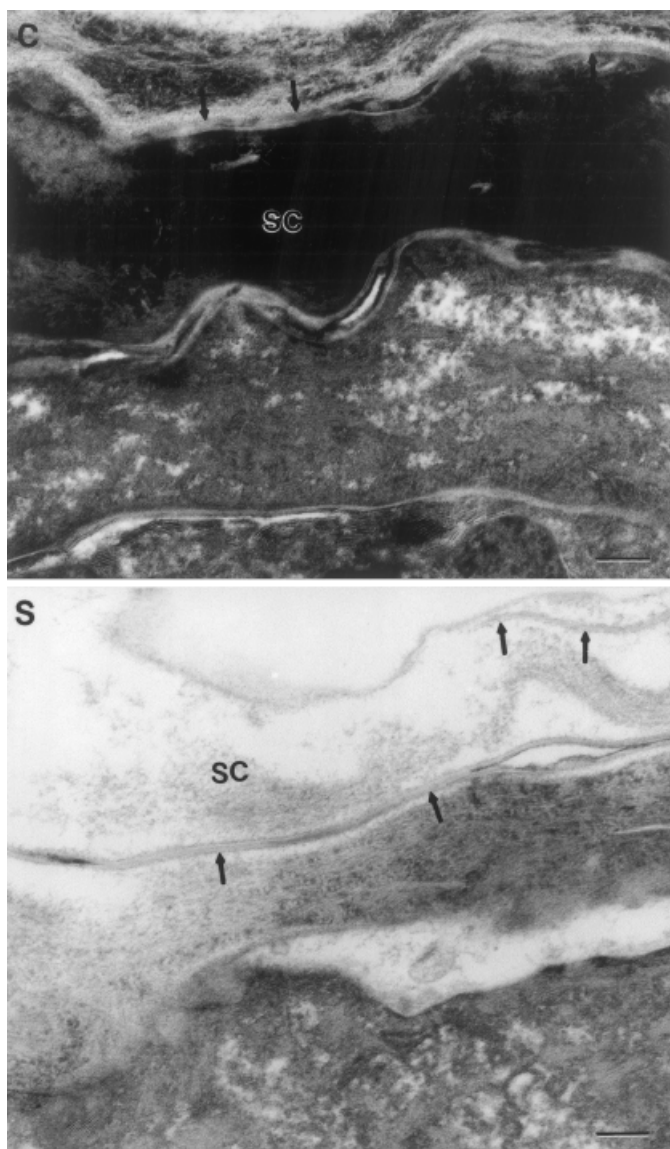


Figure 5
Psychologic stress induces a decrease in the amount of stratum corneum (SC) intercellular lamellae. Insomniac psychologic stress mice (S) showed thinner membranes (arrows) compared with control mice (C). Scale bar = 1 μ m.

abnormality in SC integrity. CD length was increased in IPS mice treated with the lipid mixture compared with IPS mice treated with the vehicle (Fig 10). These results provide further evidence that a deficiency in epidermal lipid synthesis is responsible for the abnormalities of permeability barrier homeostasis and SC integrity seen in PS animals.

Discussion

Previous studies in both rodents and in humans have shown that PS adversely affect permeability barrier homeostasis (Denda *et al*, 2000; Altemus *et al*, 2001; Garg *et al*, 2001), delaying barrier recovery following various forms of acute disruption. We determined here the basis for the adverse affects of PS on permeability homeostasis, utilizing yet another form of PS, i.e., insomnia (IPS). First, we demonstrated that IPS delayed barrier recovery kinetics, like the

previously studied form of PS. We found further that IPS epidermis displays a decreased density of LB in the SG cytosol, as well as evidence of reduced LB secretion. Recovery of barrier function is dependent on the formation and secretion of LB (Menon *et al*, 1992), which generate the lamellar lipids that mediate barrier function.

LB formation, in turn, is dependent upon epidermal lipid synthesis, i.e., inhibition of either cholesterol, fatty acid, or ceramide synthesis suppresses LB formation and delays barrier recovery following acute disruption (Feingold *et al*, 1991; Holleran *et al*, 1991; Mao-Qiang *et al*, 1993). Accordingly, we demonstrated here that the IPS-induced decrease in LB production could be attributed to reduced epidermal cholesterol, fatty acid, and ceramide synthesis. Thus, it is likely that PS, by signaling mechanisms that still remain to be elucidated, suppresses epidermal lipid synthesis, accounting for the decrease in formation of LB. We demonstrated further that a deficiency of lipids underlies the abnormality in permeability barrier homeostasis because applications of exogenous physiologic lipids, which normally have no net effect on barrier recovery (Mao-Qiang *et al*, 1995; Man *et al*, 1996; Zettersten *et al*, 1997), normalize barrier recovery kinetics in IPS animals. How IPS specifically, and PS in general signal the reduction in lipid synthesis is not known, but increased endogenous GC clearly are important.

We also identified other abnormalities in epidermal metabolism that are impaired by PS, which could result in further short- or long-term deleterious consequences for epidermal function (Denda *et al*, 1998, 2000). Specifically, both epidermal proliferation and differentiation decrease rapidly after short-term IPS. But epidermal thickness remained unchanged, presumably because of the relatively short duration of the PS, and it is likely that more sustained PS would also induce epidermal thinning, which would further impair a variety of epidermal protective functions.

IPS also produced a second important functional abnormality, a decrease in SC integrity. As previously observed for other stressors, i.e., elevated SC pH and aging, the abnormality of SC integrity was associated with a reduction in the length and number of CD (Ghadially *et al*, 1995; Reed *et al*, 1997; Fluhr *et al*, 2001). Differentiation-specific proteins of the corneocyte envelope (CE), such as desmoplakin, envoplakin, and DSG1, are CD constituents (Steinert and Marekov, 1999), and they might decrease in parallel with the three CE-linked differentiation proteins assessed here, following IPS. In this study, IPS decreased DSG1, but it is not known whether the abnormality in SC integrity can be entirely attributed to reduction in this protein alone, since other CD proteins were not assessed.

Topical applications of exogenous lipids normalized not only barrier homeostasis in IPS mice but also SC integrity, just as these lipids correct SC integrity in GC-exposed mice (Kao *et al*, 2003). Yet, multiple applications of lipids were required to correct the deficit in SC integrity, whereas a single application of lipid suffices to correct the abnormality in permeability barrier homeostasis. But the basis for the improvement in SC integrity in both PS and GC animals by provision of exogenous lipids is uncertain. It is well recognized that (i) the absolute quantities of extracellular lipids; (ii) the molar ratios of the three key lipids; as well as (iii) the

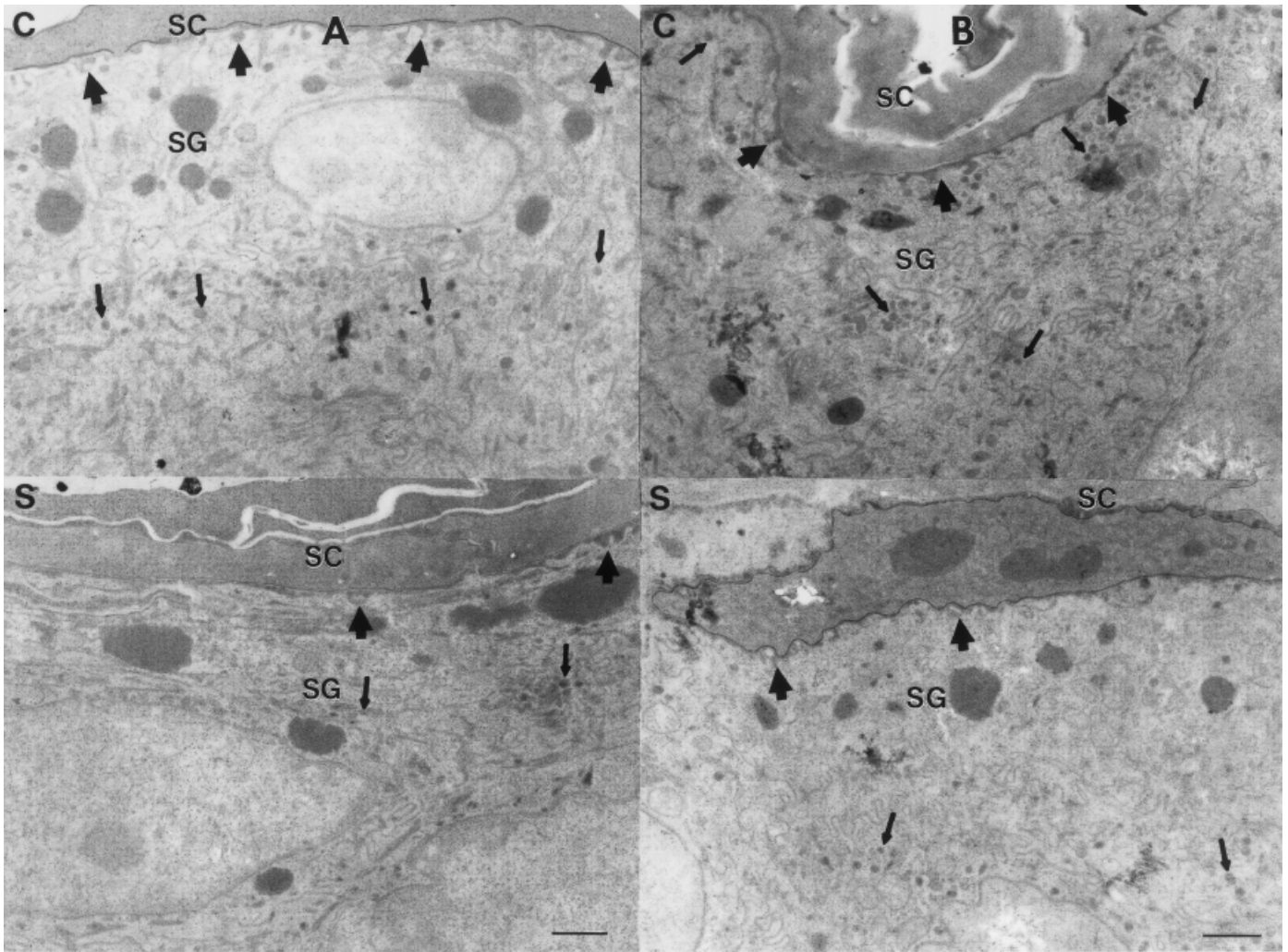


Figure 6

Psychologic stress induces a decrease of lamellar body (LB) number and secretion in the basal state and post-disruption. (A) Electron micrograph of the epidermis of insomniac psychologic stress (IPS) mice (S) shows a decrease in secretion (*wide arrows*) at the stratum corneum–stratum granulosum (SC–SG) junction and number of LB (*narrow arrows*) in the cytosol of epidermal cells compared with control (C) in the basal state. (B) Following acute barrier disruption, the decrease in secretion (*wide arrows*) and number (*narrow arrows*) of LB was still present in IPS animals. Scale bar = 2 μm .

specific composition of selected lipids in the SC interstices can influence SC desquamation rates (Williams and Elias, 1993). Thus, the IPS-induced decrease in SC lipids could potentially leave adjacent protein structures, such as CD, exposed to proteolytic degradation. For permeability barrier recovery, lipids are required acutely to allow for the optimal formation of LB, which are then secreted, forming the lamellar membranes that mediate permeability barrier function. Hence, providing the lipids in a single application immediately following barrier disruption is sufficient to restore permeability barrier recovery to normal in PS animals. In contrast, the defect in SC integrity is because of a decrease in CD density and multiple applications of lipids over hours are required to correct the defect in SC integrity.

Regardless of the lipid-based mechanisms, the decrease in SC integrity in PS mice would favor susceptibility to minor injuries, as could occur with exposure to solvents, detergents, or mechanical forces, and therefore could further perturb barrier function. Thus, increased susceptibility to barrier disruption (i.e., decreased SC integrity) is coupled with an impairment in barrier repair and the net result is

likely to have multiple, adverse clinical consequences. Yet, whereas PS abrogates a number of key functions, it did not adversely affect either SC hydration or skin surface pH, which in turn regulate several other key epidermal functions (Chuong *et al*, 2002).

The abnormalities in both permeability barrier homeostasis and SC integrity induced by PS could be mediated by increased endogenous GC (Denda *et al*, 2000). Additionally, the IPS-induced abnormalities in epidermal proliferation and differentiation are consistent with known effects of increased GC. Indeed, in the epidermis (Winter and Wilson, 1976; Sheu *et al*, 1991), the PS-induced abnormalities are mimicked by short-term topical and systemic GC treatment, which also decreased epidermal cell proliferation and thickness (Kao *et al*, 2003). Finally, the IPS-induced decrease in lipid synthesis and LB production could be GC mediated, since GC similarly inhibits these parameters (Kao *et al*, 2003), and topical treatment with exogenous lipids normalized both permeability barrier homeostasis and SC integrity in GC-treated animals, indicating a similarly important pathophysiologic role for GC in reduced lipid

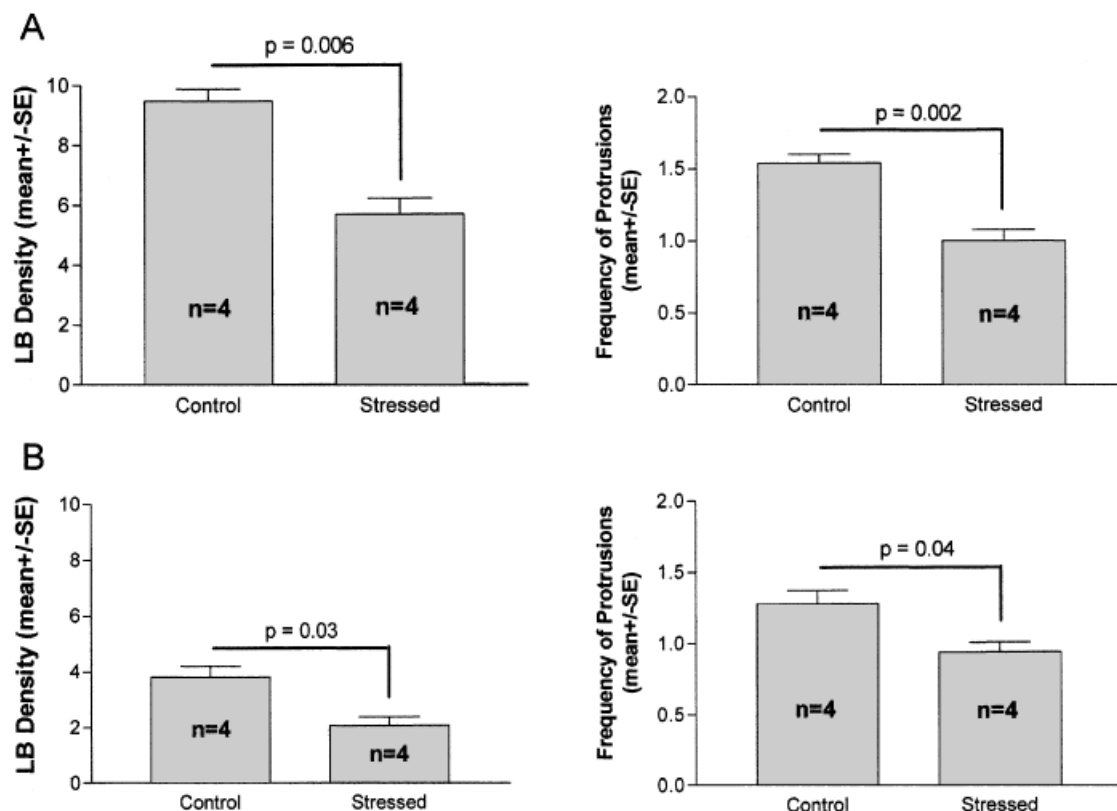


Figure 7

Effect of psychologic stress (PS) on lamellar body (LB) secretory system. The objective analysis of LB secretory system using electron microscopy demonstrated a decrease in LB formation and secretion in insomniac psychologic stress animals. LB number was measured by counting their number in the cytosol of keratinocytes and LB secretion was measured by counting the protrusion at the stratum corneum–stratum granulosum interface. PS results in a decrease in the LB secretory system both in the basal state (A) and 6 h post-barrier disruption (B). Statistical analysis was performed using unpaired Student's *t* test. Results are shown as mean ± SE (*n* = 4 animals in each group).

production (Kao *et al*, 2003). Thus, many, if not all of the changes in epidermal structure and function induced by PS are mimicked by GC treatment, suggesting that the increases in GC that are induced by PS contribute to the epidermal pathology.

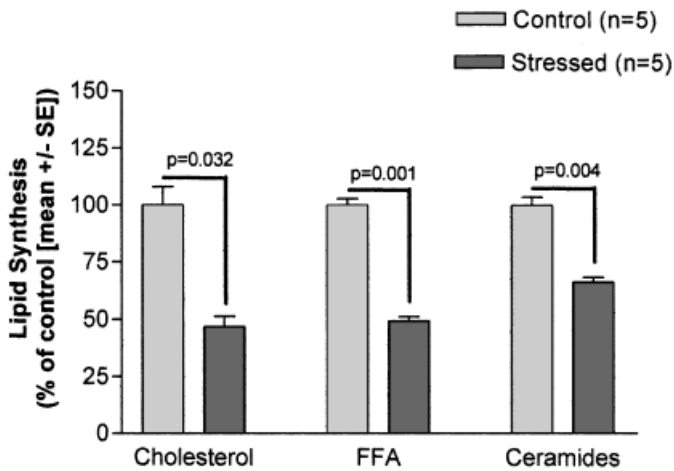
In summary, this study demonstrates that PS acutely inhibits epidermal lipid synthesis that subsequently leads to abnormalities in permeability barrier homeostasis and SC integrity. Replenishment of epidermal lipids by topical therapy reversed these abnormalities and represents a potential therapeutic modality to reduce the adverse effects of PS.

Materials and Methods

Animal model Female hairless mice (Skh1/Hr), 8–10 wk of age, were purchased from Charles River Laboratories (Wilmington, Massachusetts). All animal experiments described in this study were conducted in accordance with accepted standards of humane animal care, under protocols approved by the local animal research committee at San Francisco VA Medical center. All mice were maintained in our animal care facility in a temperature- and humidity-controlled room, and fed standard laboratory chow and tap water *ad libitum*. Prior to beginning experiments, cohorts of four animals each were kept in separate cages for at least 14 d. For the IPS group, groups of six animals at a time, each individual from a different cage, were transferred to a 12.5 cm diameter, 12.5 cm high, transparent glass jar for 42 h, and exposed to continuous visible light and radio noise. Control mice were kept in ordinary cages (four animals per cage), without continuous light and sound.

All animals continued to have free access to food and water *ad libitum*. There was no difference in body weight in the IPS *versus* control group.

Functional studies Surface pH was measured in stressed and control mice under basal conditions with a flat, glass surface electrode from Mettler-Toledo (Giessen, Germany), attached to a pH meter (PH 900; Courage & Khazaka, Cologne, Germany). SC hydration was quantitated as changes in electrical capacitance in arbitrary units (Corneometer CM 820; Courage & Khazaka) in the basal state of both stressed and control mice. The mean of three separate measurements on each animal was utilized for subsequent statistical comparison. TEWL in the basal state was measured as PPM per cm² per h with an electrolytic water analyzer (Meeco, Warrington, Pennsylvania), as described previously (Grubauer *et al*, 1989). SC integrity measures resistance to mechanical disruption, and is defined as the rate of change in TEWL with repeated tape stripping, and was determined by measurement of TEWL after each sequential stripping with 22 mm D-squame 100 tapes (CuDerm, Dallas, Texas) (Fluhr *et al*, 2001; Hachem *et al*, 2003). Barrier recovery was determined by measuring TEWL immediately after, 3, and 6 h following acute barrier disruption (TEWL levels > 4 mg per cm² per h) by tape stripping, as described previously (Grubauer *et al*, 1989; Feingold *et al*, 1990; Mao-Qiang *et al*, 1993). In some experiments, we applied 40 μL of an equimolar mixture of the three key physiologic lipids (cholesterol, free fatty acids, and ceramides [Cer2]) 1.5% in a propylene glycol:ethanol (7:3 vol/vol) vehicle *versus* vehicle alone to 4 cm² surface areas on each flank, immediately after acute barrier disruption (Kao *et al*, 2003). The effects of lipid *versus* vehicle treatment on barrier homeostasis and SC integrity were assessed in the IPS mice, as

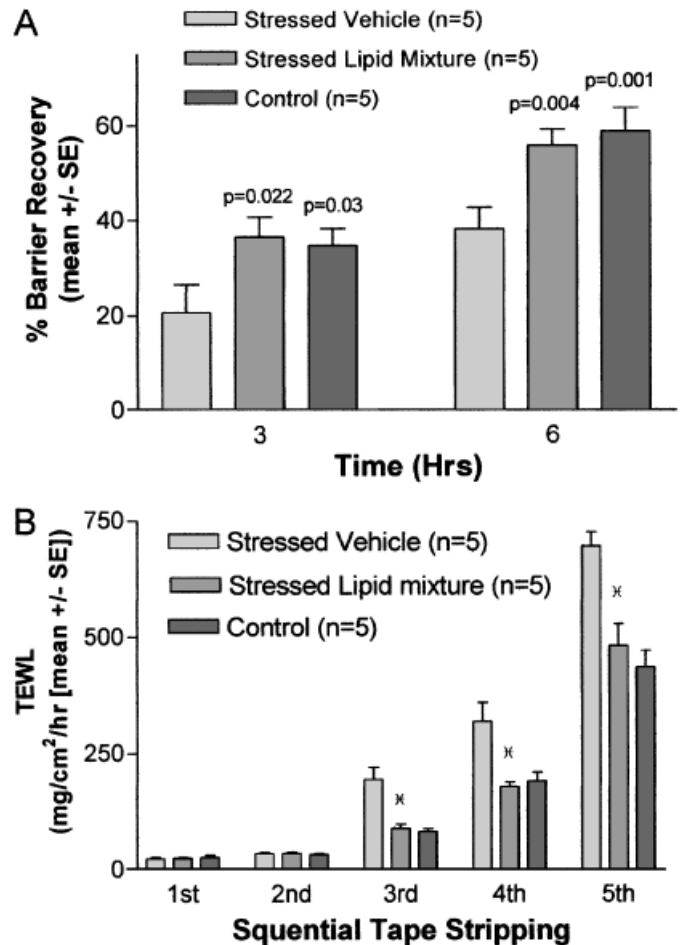
**Figure 8**

Effect of psychologic stress on epidermal lipid synthesis. After insomniac psychologic stress (IPS) was present for 42 h, skin samples were obtained for the determination of epidermal lipid synthesis in the basal state. Skin samples were incubated for 2 h with ^{14}C -acetate and incorporation into the cholesterol, free fatty acids, and ceramides was determined. The synthesis of lipids was decreased after IPS. Statistical analysis was performed using unpaired Student's *t* test. Results are expressed as mean \pm SE ($n = 5$ in each group) and control values equal 100%.

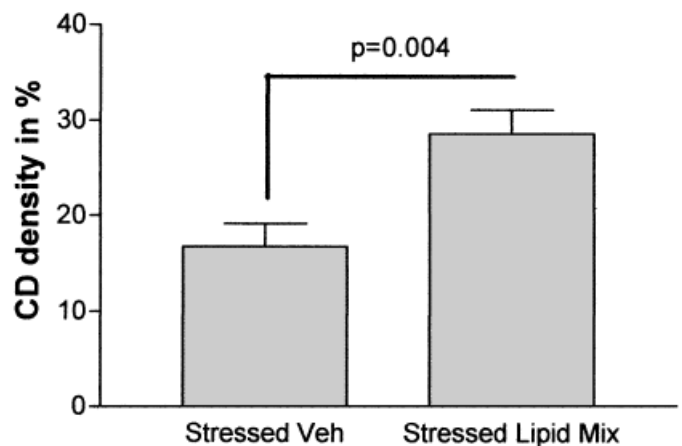
above. For SC integrity measurement, the lipid mixture was applied five times prior to stripping.

Light microscopy studies Skin biopsy samples were taken in the basal state ($n = 5$ or 6 from each group) and processed for hematoxylin and eosin (H&E) staining, PCNA immunostaining, TUNEL assay, and immunohistochemical staining for differentiation markers, including involucrin, loricrin, and filaggrin, and DSG1. Epidermal thickness was measured in 6 μm H&E-stained sections under $\times 200$ magnification, as described previously (Komuves *et al*, 2000). For epidermal DNA immunostaining we utilized a biotinylated, anti-PCNA mouse monoclonal antibody from CalTag Laboratories (Burlingame, California). Binding of the PCNA primary antibody was detected by ABC-peroxidase from Vector (Burlingame, California), utilizing diaminobenzidine as the substrate (Vector). The number of PCNA-positive cells per unit length of epidermis was compared in IPS and control mice ($n = 6$ from each group). Apoptosis was assessed by TUNEL assay in deparaffinized sections, treated first with 0.5% sodium tetraborohydrate for 30 min, using an *in situ* Cell Death Detection Kit from Boehringer-Mannheim (Indianapolis, Indiana) (Komuves *et al*, 2000). Affinity-purified rabbit antibodies specific for mouse involucrin, loricrin, and filaggrin were obtained from BabCo (Richmond, California); DSG1 was a gift from Dr John Stanley, University of Pennsylvania. Affinity-purified biotinylated goat anti-rabbit IgG was purchased from Vector. Immunohistochemical staining for the differentiation proteins and DSG1 was detected by the ABC-peroxidase method, as above (Komuves *et al*, 2000). Negative controls without primary antibodies showed no immunolabeling.

EM studies Skin biopsy samples were taken in the basal state and 6 h after tape stripping ($n = 6$ from each group), at which time point the differences between the IPS and control animals were most pronounced. Samples were minced to less than 0.5 mm^3 , fixed in modified Karnovsky's fixative overnight, and post-fixed in 0.5% ruthenium tetroxide (RuO_4) and 2% aqueous osmium tetroxide (OsO_4) containing 1.5% potassium ferrocyanide (Hou *et al*, 1991; Menon *et al*, 1992). After post-fixation, all samples were dehydrated in graded ethanol solutions and embedded in an Epon-epoxy mixture. Ultrathin sections were examined, with or without further

**Figure 9**

Effect of topical lipids on skin barrier recovery (A) and stratum corneum (SC) integrity (B) in psychologic stress (PS) animals. (A) Immediately after tape stripping, insomniac psychologic stress (IPS) animals were topically treated once with a lipid mixture containing cholesterol, fatty acids, and ceramides. Barrier recovery was determined at 3 and 6 h after disruption. Statistical analysis was performed using repeated ANOVA. Results are expressed as mean \pm SE ($n = 5$ in each group). (B) During PS, the lipid mixture was applied three times a day. The lipid mixture improved the abnormality in SC integrity induced by IPS. * $p < 0.05$ IPS vehicle versus IPS lipid mixture.

**Figure 10**

Effect of topical lipids on corneodesmosome (CD) in psychologic stress animals. Topical lipids restored CD in insomniac psychologic stress (IPS) animal compared with vehicle-treated IPS animal. Statistical analysis was performed using unpaired Student's *t* test. Results are shown as mean \pm SE ($n = 4$ animals in each group).

lead citrate contrasting, in Zeiss 10A electron microscope (Carl Zeiss, Thornwood, New York), operated at 60 kV.

Quantitative EM analysis In order to exclude subjective bias in these morphologic studies, we quantitated both CD and LB number (= density) and secretion in EM pictures by an objective method. We used four or five EM pictures taken at low magnification ($\times 5000$) from each sample to cover large sample areas; to further diminish bias; and to improve statistical sampling.

LB quantitation The numbers of protrusions (= invagination along the SC–SG interface) were quantitated, and assessed planimetrically as the number per unit length of SC–SG interface. To assess LB densities, LB images in the cytosol of the uppermost two layers of the SG were counted and expressed as average number per unit area of cytosol.

CD quantitation We measured CD length at random from the first and second cell layers of the lower SC. The ratio of the total length of intact CD to the total length of cornified envelopes was determined by planimetry, as described previously (Morris, 2000; Kao *et al*, 2001; Hachem *et al*, 2003).

Lipid synthesis Full-thickness skin samples were obtained from anesthetized, stressed, and control mice ($n = 5$) under basal conditions, i.e., after 42 h of continuous IPS or control conditions. The skin samples were incubated for 2 h in a solution containing 10 mM ethylenediamine tetra-acetic acid in Dulbecco's phosphate-buffered saline, calcium and magnesium free, containing 25 μCi ^{14}C -acetate at 37°C . After incubations, the epidermis was separated from the dermis, and the incorporation of ^{14}C -acetate into cholesterol, fatty acids, and ceramides was determined after saponification, extraction, and thin-layer chromatography (Kao *et al*, 2003). Individual lipid bands were scraped from the plates, incubated in Scintisafe 30% (Fisher Scientific, Santa Clara, California), and counted in a Beckman LS 1800 scintillation counter (Beckman, Fullerton, California) (Menon *et al*, 1985; Feingold and Elias, 1988; Holleran *et al*, 1991).

Statistical analyses Data were expressed as the means \pm SE. Statistical analyses were performed using paired and unpaired Student's *t* tests and repeated ANOVA.

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Supplementary Material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23589/JID23589sm.htm>

Figure S1

Effect of psychological stress on epidermal proliferation.

Figure S2

Effect of psychological stress on epidermal differentiation.

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